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SOMATIC EMBRYOGENESIS AND PROTOPLAST ISOLATION AND CULTURE IN PAPAYA (CARICA PAPAYA L.)

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SOMATIC EMBRYOGENESIS AND PROTOPLAST ISOLATION AND CULTURE IN PAPAYA (*CARICA PAPAYA* L.)

By

AGUS SUTANTO

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Agriculture Universiti Putra Malaysia

September 2001

Dedicated to:

My beloved wife Susilowati

My dearest son Ikhsan Fitrianto

Departed soul of my father Munari

BIODATA OF THE AUTHOR

The author was born on 3 August 1967 in Malang, East Java, Indonesia. He graduated from primary school, junior high school and senior high school in 1980, 1983 and 1985 respectively. In 1985, he continued his study at Brawijaya University Malang. He began to study tissue culture in 1990 and completed his study in the university in 1991 with specialization in tissue culture.

After graduation, he worked at Tlekung Horticultural Research Station, Malang, as a researcher on tissue culture of citrus, apple and mangosteen. He participated in a Banana Tissue Culture Course at Taiwan Banana Research Institute (TBRI) and Banana Breeding Workshop at Malaysian Research and Development Institute (MARDI), both in 1994. In 1995, due to reorganization of the Department of Agriculture, he shifted to Solok Research Institute for Fruits, West Sumatera. He worked on banana breeding and tissue culture of citrus, mango and papaya. In 1996, under the collaboration of the Department of Agriculture and International Network for the Improvement of Banana and Plantain (INIBAP), he went to Maluku islands to collect *Musa* germplasms.

In 1997, he was recommended by the Agency for Agriculture Research and Development (AARD), Department of Agriculture, Republic of Indonesia, to continue his study and began his Master of Science program in November 1998 at the Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia in the field of Agricultural Biotechnology.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

SOMATIC EMBRYOGENESIS AND PROTOPLAST ISOLATION AND CULTURE IN PAPAYA (*CARICA PAPAYA* L.)

By

AGUS SUTANTO

September 2001

Chairman : Dr. Maheran Abdul Aziz

Faculty : Agriculture

This study was carried out with the main objective of establishing somatic embryo production in papaya that may be used for further genetic improvement of the crop. The specific objectives were to induce somatic embryogenesis from immature zygotic embryos, to establish cell suspension culture of somatic embryos and to isolate and culture protoplasts from *in vitro* leaves.

The first experiment studied the effect of growth regulators on the induction and regeneration of somatic embryos from immature zygotic embryos. The combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) at various concentrations were assessed in order to determine the best combination for somatic embryogenesis. The experiment was conducted in a Completely Randomized Design.

The results showed that higher 2,4-D with or without BAP in the medium increased the percentage of somatic embryo formation and the number of somatic embryos per explant. MS medium supplemented with 5.0 mg/L 2,4-D promoted the highest

percentage of somatic embryogenesis (74.55%), while the combination of 1.0 mg/L 2,4-D and 0.01 mg/L BAP produced the highest percentage of callus formation (52.58%). The highest number of somatic embryos per explant (66.61) was obtained when 5.0 mg/L 2,4-D and 0.01 mg/L BAP were added into MS medium. For germination of somatic embryos, the result was very inconsistent; nevertheless, the best treatment for plantlet formation was MS medium without growth hormone (N_0B_0).

The second experiment was the establishment of cell suspension culture of somatic embryos. Four weeks after the transfer of somatic embryos into liquid MS medium containing 2.0 mg/L 2,4-D, pro-embryogenic masses (PEMs) were formed in the suspension. Maturation of embryos was achieved on transferring the heart-shaped embryos to liquid MS medium without growth regulator. Germination of somatic embryos occurred following the subculture of cotyledonary embryos from liquid MS medium with 0.2 mg/L BAP and 0.02 mg/L naphthalene acetic acid (NAA) to solid hormone-free MS medium.

The third experiment was the isolation, purification and culture of protoplasts. *In vitro* leaves derived from shoot tips cultured on medium with 0.05 mg/L or 0.10 mg/L BAP were used as the source of protoplasts. Enzyme solution containing cell protoplast washing (CPW) salts, 0.6% (w/v) Macerozyme R-10, 2.0% (w/v) Cellulase R-10, 0.2% (w/v) Driselase and 9.0% (w/v) mannitol was used for protoplast isolation. Protoplasts were purified using two methods of purification; filtration followed by repeated resuspension, and floatation on dense sucrose solution.

The results showed that the highest protoplast yield and percentage of viable protoplasts were obtained when *in vitro* leaves from shoot tip culture placed on MS medium with 0.05 mg/L BAP were used as the source of protoplasts and the protoplasts purified by floatation on dense sucrose solution. Three days following the protoplast culture in modified KM8p medium, first division of protoplast-derived cells was observed, but they failed to grow further.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

EMBRIOGENESIS SOMA DAN PENGASINGAN SERTA KULTUR PROTOPLAS BAGI TANAMAN BETIK (*CARICA PAPAYA* L.)

Oleh

AGUS SUTANTO

September 2001

Pengerusi : Dr. Maheran Abdul Aziz

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Penyelidikan ini dilaksanakan dengan objektif utama untuk mewujudkan produksi embrio soma betik, yang boleh digunakan untuk perbaikan genetik tanaman tersebut. Objektif khas adalah induksi dan regenerasi embrio soma daripada embrio zigotik belum matang, mewujudkan kultur ampaian sel daripada embrio soma dan isolasi serta kultur protoplas dari daun yang dikulturkan secara *in vitro*.

Eksperimen pertama mengkaji kesan pengawalatur tumbesaran terhadap induksi dan regenerasi embrio soma daripada embrio zigotik belum matang. Kombinasi asid diklorofenoksi asetik (2,4-D) dan 6-bensilaminopurina (BAP) pada beberapa kepekatan dicuba untuk mengenalpasti kombinasi terbaik untuk induksi embrio soma. Kajian ini dijalankan dengan menggunakan Rekabentuk Rawak Lengkap (CRD).

Hasil kajian menunjukkan bahawa media yang mengandungi kepekatan 2,4-D yang tinggi dengan atau tanpa BAP merangsang dan meningkatkan peratus pembentukan embryo soma dan bilangan embryo soma per eksplan. Media MS dengan 5.0 mg/L

2,4-D merangsang peratus pembentukan embrio soma tertinggi (74.55%), sementara kombinasi 1.0 mg/L 2,4-D dan 0.01 mg/L BAP menghasilkan peratus pembentukan kalus tertinggi (52.58%). Bilangan embrio soma per eksplan tertinggi diperolehi apabila 5.0 mg/L 2,4-D dan 0.01 mg/L BAP ditambahkan ke dalam media MS. Untuk percambahan embrio soma, hasil kajian tidak konsisten, tetapi rawatan terbaik untuk pembentukan planlet adalah media MS tanpa pengawalatur tumbesaran (N_0B_0).

Eksperimen kedua adalah perwujudan kultur ampaian sel daripada embrio soma. Empat minggu setelah embrio soma dipindah ke dalam media cecair MS yang mengandungi 2.0 mg/L 2,4-D, 'pro-embryogenic masses' (PEM) terbentuk di dalam kultur ampaian. Kematangan embrio dicapai dengan memindahkan embrio berbentuk hati ke dalam media MS tanpa pengawalatur tumbesaran. Percambahan embrio soma berlaku berikutan pemindahan embrio berkotiledon dari media cecair MS dengan 0.2 mg/L BAP dan 0.02 mg/L NAA ke media pepejal MS tanpa pengawalatur tumbesaran.

Eksperimen ketiga adalah pengasingan, penulenan dan kultur protoplas. Daun *in vitro* daripada tunas pucuk yang dikultur dalam media MS dengan 0.05 mg/L atau 0.10 mg/L BAP digunakan sebagai sumber protoplas. Larutan enzim yang mengandungi garam penyucian sel protoplas (CPW), 0.6% (w/v) Macerozyme R-10, 2.0% (w/v) Cellulase R-10, 0.2% (w/v) Driselase dan 9.0% (w/v) mannitol digunakan untuk isolasi protoplas. Penulenan protoplas menggunakan dua kaedah, iaitu penurasan yang diikuti dengan pengampaian semula, dan sistem pengapungan pada larutan sukrosa ketumpatan tinggi.

Kajian menunjukkan hasil protoplas dan peratus protoplas bernas yang tertinggi diperolehi apabila daun yang berasal daripada tunas pucuk yang dikulturkan pada media MS dengan 0.05 mg/L BAP digunakan sebagai sumber protoplas dan penulenan protoplas menggunakan sistem pengapungan pada larutan sukrosa ketumpatan tinggi. Tiga hari setelah protoplas dikultur dalam media KM8p yang telah diubahsuai kandungannya, sel-sel yang terbentuk dari protoplas mengalami pembahagian pertama, tetapi gagal untuk meneruskan pertumbuhan.

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I certify that an Examination Committee met on 11th September 2001 to conduct the final examination of Agus Sutanto on his Master of Science thesis entitled "Somatic Embryogenesis and Protoplast Isolation and Culture in Papaya (*Carica papaya* L.)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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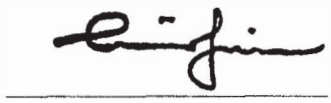
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.



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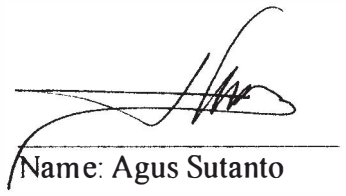
DECLARATION

I hereby declare that this thesis is based on my original work except quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



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2,4-D	2,4-dichlorophenoxyacetic acid
AARD	Agency for Agriculture Research and Development
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic
ANOVA	analysis of variance
Arcsin	$\sin^{-1}(X)$
ASEAN	Association of South East Asia Nations
BAP	6-benzylaminopurine
carETR1	ethylene response 1 gene isolated from *Carica*
cDNA	copy of deoxyribonucleic acid
CPW	cell and protoplast washing medium (Frearson *et al.* 1973)
CRD	completely randomized design
cv.	cultivar
DMRT	duncan multiple range test
e.g.	exempli gratia (for example)
et al.	*et alia*
etc.	et cetera
F_1	first filial generation
FDA	fluorescein diacetate
Fe-EDTA	iron ethylene diamine tetraacetic acid
g/L	gram per liter
h.	hours
IAA	indole acetic acid
IBA	indole butyric acid
i.e.	*id est* (that is)
IEDCs	induced embryogenic determined cells
INIBAP	International Network for the Improvement of Banana and Plantain
KCal	kilo calories

KM8p	Kao and Michayluk (1975) basal medium
M	molar
MARDI	Malaysian Agriculture Research and Development Institute
mg/L	milligram per litre
mM	millimolar
MS	Murashige and Skoog (1962) basal medium
NAA	naphthalene acetic acid
NCSS	Number Cruncher Statistical System
NN	Nitch and Nitch (1969) basal medium
PEDCs	pre-embryogenic determined cells
PEMs	pro-embryogenic masses
pH	$^{-}\log(H^{+})$
PRV	papaya ring spot virus
Ri	root-induction
RITA	*Récipient à Immersion Temporarire Automatisé*
RNA	ribonucleic acid
rpm	rotary per minute
sp.	species
TBRI	Taiwan Banana Research Institute
Ti	tumor-induction
TIBA	2,3,5 tri-iodobenzoic acid
USA	United State of America
UV	ultra violet light
v/v	volume to volume
VBCB	virus bintik cincin betik
w/v	weight to volume
WPM	woody plant basal medium (Lloyd and McCown, 1981)
x g	x gravity (relative centrifugal force)
%	percent
$\sqrt{}$	square root

α	level of significance
µg	microgram
µm	micrometer

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Papaya is an important fruit in South East Asia countries such as Thailand, Indonesia, the Philippines and Malaysia. The ripe fruit is usually eaten as dessert, processed into jams, juice, and soft drink and as fillers for sauces. Besides supplying food, an important product of papaya is papain, which is in great demand in the international market, particularly United Kingdom and USA. Papain is used in meat tenderizing, manufacture of chewing gum and cosmetics, tannin hides, degumming of natural silk and to give shrink-resistance to wool. In South East Asia, papain is produced commercially only in the Philippines (Rohani, 1994).

Papaya is a very wholesome fruit and provides a cheap source of vitamins and minerals in the daily diet of the people. The fruit contains 1.0-1.5% protein and a plentiful source of carotene (1,160-2,431 μg per 100 g edible portion), the precursor of vitamin A. It is also a good source of vitamin C (69-71 mg/100 g) and mineral such as calcium (11-31 mg/100 g) and potassium (39-337 mg/100 g). The fruit is also popular with dieters since it is low in fat (0.1%), carbohydrate (7-13%) and calories (35-59 KCal/100 g) (Rohani, 1994).

The popularity of papaya fruit and the ease with which it can be propagated has made it ubiquitous in tropical and subtropical region Local papayas are